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APPLICATION

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TITLE: MUTATIONS ASSOCIATED WITH IRON DISORDERS

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MUTATIONS ASSOCIATED WITH IRON DISORDERS

Background of the Invention

Hemochromatosis is the most common progressive (and sometimes fatal) genetic disease in people of European descent. Hemochromatosis is a disease state characterized by an inappropriate increase in intestinal iron absorption. The increase can result in deposition of iron in organs such as the liver, pancreas, heart, and pituitary. Such iron deposition can lead to tissue damage and functional impairment of the organs.

In some populations, 60-100% of cases are attributable to homozygosity for a missense mutation at C282Y in the <u>H</u>istocompatibility iron (<u>Fe</u>) loading (HFE) gene, a major histocompatibility (MHC) non-classical class I gene located on chromosome 6p. Some patients are compound heterozygotes for C282Y and another mutation at H63D.

Summary of the Invention

The invention is based on the discovery of novel mutations which are associated with aberrant iron metabolims, absorption, or storage, or in advanced cases, clinical hemochromatosis. Accordingly, the invention features a method of diagnosing an iron disorder, e.g., hemochromatosis or a genetic susceptibility to developing such a disorder, in a mammal by determining the presence of a mutation in exon 2 of an HFE nucleic acid. The mutation is not a C→G missense mutation at position 187 of SEQ ID NO:1 which leads to a H63D substitution. The nucleic acid is an RNA or DNA molecule in a biological sample taken from the mammal, e.g. a human patient, to be tested. The presence of the mutation is indicative of the disorder or a genetic susceptibility to developing it. An iron disorder is characterized by an aberrant serum iron level, ferritin level, or percent saturation of transferrin compared to the

level associated with a normal control individual. An iron overload disorder is characterized by abnormally high iron absorption compared to a normal control individual. Clinical hemochromatosis is defined by an elevated fasting transferrin saturation level of greater than 45% saturation.

For example, the mutation is a missense mutation at nucleotide 314 of SEQ ID NO:1 such as 314C which leads to the expression of mutant HFE gene product with amino acid substitution I105T. The I105T mutation is located in the α1 helix of the HFE protein and participates in a hydrophobic pocket (the "F" pocket). The alpha helix structure of the α1 domain spans residues S80 to N108, inclusive. The I105T mutation is associated with an iron overload disorder.

Table 1: Human HFE cDNA sequence

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 atgggcccg cgagccaggc
cggcgcttct cctcctgatg cttttgcaga ccgcggtcct gcaggggcgc ttgctgcgtt
cacactctct gcactacctc ttcatgggtg cctcagagca ggaccttggt ctttccttgt
ttgaagcttt gggctacgtg gatgaccagc tgttcgtgtt ctatgatcat gagagtcgcc
                                              H63D   S65C
gtgtggagcc ccgaactcca tgggtttcca gtagaatttc aagccagatg tggctgcagc
tgagtcagag tctgaaaggg tgggatcaca tgttcactgt tgacttctgg actattatgg
                 G93R                                  I105T
aaaatcacaa ccacagcaag gagtcccaca ccctgcaggt catcctgggc tgtgaaatgc
aagaagacaa cagtaccgag ggctactgga agtacgggta tgatgggcag gaccaccttg
aattctgccc tgacacactg gattggagag cagcagaacc cagggcctgg cccaccaagc
tggagtggga aaggcacaag attcgggcca ggcagaacag ggcctacctg gagagggact
gccctgcaca gctgcagcag ttgctggagc tggggagagg tgttttggac caacaagtgc
ctcctttggt gaaggtgaca catcatgtga cctcttcagt gaccactcta cggtgtcggg
ccttgaacta ctacccccag aacatcacca tgaagtggct gaaggataag cagccaatgg
atgccaagga gttcgaacct aaagacgtat tgcccaatgg ggatgggacc taccagggct
ggataacctt ggctgtaccc cctggggaag agcagagata tacgtgccag gtggagcacc
caggcctgga tcagcccctc attgtgatct gggagccctc accgtctggc accctagtca
ttggagtcat cagtggaatt gctgtttttg tcgtcatctt gttcattgga attttgttca
taatattaag gaagaggcag ggttcaagag gagccatggg gcactacgtc ttagctgaac
gtgagtgaca cgcagcctgc agactcactg tgggaaggag acaaaactag agactcaaag
agggagtgca tttatgagct cttcatgttt caggagagag ttgaacctaa acatagaaat
tgcctgacga actccttgat tttagccttc tctgttcatt tcctcaaaaa gatttcccca
tttaggtttc tgagttcctg catgccggtg atccctagct gtgacctctc ccctggaact
gtctctcatg aacctcaagc tgcatctaga ggcttccttc atttcctccg tcacctcaga
gacatacacc tatgtcattt catttcctat ttttggaaga ggactcctta aatttggggg
acttacatga ttcattttaa catctgagaa aagctttgaa ccctgggacg tggctagtca
taaccttacc agatttttac acatgtatct atgcattttc tggacccgtt caactttcc
tttgaatcct ctctctgtgt tacccagtaa ctcatctgtc accaagcctt ggggattctt
ccatctgatt gtgatgtgag ttgcacagct atgaaggctg tgcactgcac gaatggaaga
ggcacctgtc ccagaaaaag catcatggct atctgtgggt agtatgatgg gtgtttttag
caggtaggag gcaaatatct tgaaaggggt tgtgaagagg tgttttttct aattggcatg
aaggtgtcat acagatttgc aaagtttaat ggtgccttca tttgggatgc tactctagta
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ttccagacct gaagaatcac aataattttc tacctggtct ctccttgttc tgataatgaa
aattatgata aggatgataa aagcacttac ttcgtgtccg actcttctga gcacctactt
acatgcatta ctgcatgcac ttcttacaat aattctatga gataggtact attatcccca
tttctttttt aaatgaagaa agtgaagtag gccgggcacg gtggctcgcg cctgtggtcc
cagggtgctg agattgcagg tgtgagccac cctgcccagc cgtcaaaaga gtcttaatat
atatatccag atggcatgtg tttactttat gttactacat gcacttggct gcataaatgt
ggtacaacca ttctgtcttg aagggcaggt gcttcaggat accatataca gctcagaagt
ttcttcttta ggcattaaat tttagcaaag atatctcatc tcttctttta aaccattttc
tttttttgtg gttagaaaag ttatgtagaa aaaagtaaat gtgatttacg ctcattgtag
aaaagctata aaatgaatac aattaaagct gttatttaat tagccagtga aaaactatta
acaacttgtc tattacctgt tagtattatt gttgcattaa aaatgcatat actttaataa
atgtacattg tattgtaaaa aaaaaaa

(SEQ ID NO:1; GENBANK® Accession No. U60319)

Table 2: Human HFE gene product

MGPRARPALLLLMLLQTAVLQG

RLLRSHSLHYLFMGASEQDLGLSLFEALGYVDDQLFVFYD**H**E**S**RRVEPRTPWVSSRISSQMWLQLSQSLK**G**WDHMFTVDFWT**I**MENHNHSKESHTLQVILGCEMQEDNSTEGYWKYGYDGQDHLEFCPDTLDWRAAEPRAWPTKLEWERHKIRARQNRAYLERDCPAQLQQLLELGRGVLDQQVPPLVKVTHHVTSSVTTLRCRALNYYPQNITMKWLKDKQPMDAKEFEPKDVLPNGDGTYQGWITLAVPPGEEQRYTCQVEHPGLDQPLIVIWEPSPSGTLVIGVISGIAVFVVILFIGILFIILRKRQGSRGAMGHYVLAERE (SEQ ID NO: 2; GENBANK® Accession No. U60319)

Residues 1-22 = leader sequence; α1 domain underlined; residues 63, 65, 93, and 105 indicated in bold type)

Other mutations include nucleotide 277 of SEQ ID NO: 1, e.g., 277C which leads to expression of mutant HFE gene product G93R and one at nucleotide 193 of SEQ ID NO: 1, e.g., 193T, which leads to expression of mutant HFE gene product S65C.

Any biological sample containing an HFE nucleic acid or gene product is suitable for the diagnostic methods described herein. For example, the biological sample to be analyzed is whole blood, cord blood, serum, saliva, buccal tissue, plasma, effusions, ascites, urine, stool, semen, liver tissue, kidney tissue, cervical tissue, cells in amniotic fluid, cerebrospinal fluid, hair or tears. Prenatal testing can be done using methods used in the art, e.g., amniocentesis or chorionic villa sampling. Preferably, the biological sample is one that can be non-

invasively obtained, e.g., cells in saliva or from hair follicles.

The assay is also used to screen individuals prior to donating blood to blood banks and to test organ tissue, e.g., a donor liver, prior to transplantation into a recipient patient. Both donors and recipients are screened.

In some cases, a nucleic acid is amplified prior to detecting a mutation. The nucleic acid is amplified using a first oligonucleotide primer which is 5' to exon 2 and a second oligonucleotide primer is 3' to exon 2. To detect mutation at nucleotide 314 of SEQ ID NO: 1, a first oligonucleotide primer which is 5' to nucleotide 314 and a second oligonucleotide primer which is 3' to nucleotide 314 is used in a standard amplification procedure such as polymerase chain reaction (PCR). To amplify a nucleic acid containing nucleotide 277 of SEQ ID NO: 1, a first oligonucleotide primer which is 5' to nucleotide 277 and a second oligonucleotide primer which is 3' to nucleotide 277 is used. Similarly, a nucleic acid containing nucleotide 193 of SEQ ID NO:1 is amplified using primers which flank that nucleotide. For example, for nucleotide 277, the first primer has a nucleotide sequence of SEQ ID NO: 3 and said second oligonucleotide primer has a nucleotide sequence of SEQ ID NO: 4, or the first primer has a nucleotide sequence of SEQ ID NO: 15 and said second oligonucleotide primer has a nucleotide sequence of SEQ ID NO: 16. Table 3, below, shows examples of primer pairs for amplification of nucleic acids in exons and introns of the HFE gene.

<u>Table 3</u>

	I. PRIMERS USED FOR AMPLIFICATION	
Target DNA	Forward Primer	Reverse Primer
Exon 2	CCTCCTACTACACATGGTTAAGG	GCTCTGACAACCTCAGGAAGG
	(SEQ ID NO: 3)	(SEQ ID NO: 4)
Exon 3	GGTGGAAATAGGGACCTATTCC	CACTCTGCCACTAGACTATAGG
	(SEQ ID NO: 5)	(SEQ ID NO: 6)
Exon 4	GTTCCAGTCTTCCTGGCAAGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 7)	(SEQ ID NO: 8)
RT-PCR	AAAGGATCCACCATGGGCCCGCGAGCCAGG	GTGAGTCTGCAGGCTGCGTG
	(SEQ ID NO: 9)	(SEQ ID NO: 10)
Intron 4	GTTCCAGTCTTCCTGGCAAGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 11)	(SEQ ID NO: 12)
Intron 5	GTTCCAGTCTTCCTGGCAAGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 13)	(SEQ ID NO: 14)
	II. PRIMERS USED FOR AMPLIFICATION	
Target DNA	Forward Primer	Reverse Primer
Exon 2	GTGTGGAGCCTCAACATCCTG	ACAAGACCTCAGACTTCCAGC
	(SEQ ID NO: 15)	(SEQ ID NO: 16)
Exon 3	GGTGGAAATAGGGACCTATTCC	CACTCTGCCACTAGAGTATAGG
	(SEQ ID NO: 17)	(SEQ ID NO: 18)
Exon 4	GTTCCAGTCTTCCTGGCAAGG	TTACCTCCTCAGGCACTCCTC
	(SEQ ID NO: 19)	(SEQ ID NO: 20)
RT-PCR	AAAGGATCCACCATGGGCCCGCGAGCCAGG	GTGAGTCTGCAGGCTGCGTG
	(SEQ ID NO: 21)	(SEQ ID NO: 22)
Intron 4	TGCCTGAGGAGGTAATTATGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 23)	(SEQ ID NO: 24)
Intron 5	TGCCTGAGGAGGTAATTATGG	AAATGCTTCCCATGGATGCCAG
	(SEQ ID NO: 25)	(SEQ ID NO: 26)

Mutations in introns of the HFE gene have now been associated with iron disorders and/or hemochromatosis. By "exon" is meant a segment of a gene the sequence of which is represented in a mature RNA product, and by "intron" is meant a segment of a gene the sequence of which is not represented in a mature RNA product. An intron is a part of a primary nuclear transcript which is subsequently spliced out to produce a mature RNA product, i.e., a mRNA, which is then transported to the cytoplasm. A method of diagnosing an iron disorder or a genetic susceptibility to developing the disorder is carried out by determining the presence or absence of a mutation in an intron of HFE genomic DNA in a biological sample. The presence of the mutation is indicative of the disorder or a genetic susceptibility to developing the disorder. The presence of a mutation in an intron is a marker for an exon mutation, e.g., a mutation in intron 4, e.g., at nucleotide 6884 of SEQ ID NO:27 is associated with the S65C mutation in exon 2. A mutation in intron 5, e.g., at nucleotide 7055 of SEQ ID NO:27 is associated with hemochromatosis. In some cases, intron mutations may adversely affect proper splicing of exons or may alter regulatory signals. Preferably, the intron 4 mutation is 6884C and the intron 5 mutation is 7055G. To amplify nucleic acid molecule containing nucleotide 6884 or 7055, primers which flank that nucleotide, e.g., those described in Table 3, are used according to standard methods. Nucleic acid-based diagnostic methods may or may not include a step of amplification to increase the number of copies of the nucleic acid to be analyzed. To detect a mutation in intron 4, a patient-derived nucleic acid may be amplified using a first oligonucleotide primer which is 5' to intron 4 and a second oligonucleotide primer which is 3'

to intron 4, and to detect a mutation in intron 5, the nucleic acid may be amplified using a first oligonucleotide primer which is 5' to intron 5 and a second oligonucleotide primer which is 3' to intron 5 (see, e.g., Table 3).

In addition to nucleic acid-based diagnostic methods, the invention includes a method of diagnosing an iron overload disorder or a genetic susceptibility thereto by determining the presence of a mutation in a HFE gene product in a biological sample. For example, the mutation results in a decrease in intramolecular salt bridge formation in the mutant HFE gene product compared to salt bridge formation in a wild type HFE gene product. The mutation which affects salt bridge formation is at or proximal to residue 63 of SEQ ID NO:2, but is not amino acid substitution H63D. Preferably, the mutation is between residues 23-113, inclusive of SEQ ID NO:2 (Table 2), more preferably, it is between residues 90-100, inclusive, of SEQ ID NO:2, more preferably, it is between residues 58-68, inclusive, of SEQ ID NO:2, and most preferably, the mutation is amino acid substitution S65C. Alternatively, the mutation which affects salt bridge formation is a mutation, e.g., an amino acid substitution at residue 95 or proximal to residue 95 of SEQ ID NO:2. Preferably, the mutation is G93R. Such an HFE mutation is detected by immunoassay or any other ligand binding assay such as binding of the HFE gene product to a transferrin receptor. Mutations are also detected by amino acid sequencing, analysis of the structural conformation of the protein, or by altered binding to a carbohydrate or peptide mimetope.

A mutation indicative of an iron disorder or a genetic susceptibility to developing such a disorder is located in the α1 helix (e.g., which spans residues 80-108, inclusive, of SEQ ID NO:2) of an HFE gene product. The

mutation may be an addition, deletion, or substitution of an amino acid in the wild type sequence. For example, the mutant HFE gene product contains the amino acid substitution I105T or G93R or in the loop of the β sheet of the HFE molecule, e.g., mutation S65C

Isolated nucleic acids encoding a mutated HFE gene products (and nucleic acids with nucleotide sequences complementary to such coding sequences) are also within the invention. Also included are nucleic acids which are at least 12 but less than 100 nucleotides in length. An isolated nucleic acid molecule is a nucleic acid molecule that is separated from the 5' and 3' sequences with which it is immediately contiguous in the naturally occurring genome of an organism. "Isolated" nucleic acid molecules include nucleic acid molecules which are not naturally occurring. For example, an isolated nucleic acid is one that has been amplified *in vitro*, e.g, by PCR; recombinantly produced; purified, e.g., by enzyme cleavage and gel separation; or chemically synthesized. For example, the restriction enzyme, Bst4C I (Sib Enzyme Limited, Novosibirsk, Russia), can be used to detect the G93R mutation (point mutation 277C); this enzyme cuts the mutated HFE nucleic acid but not the wild type HFE nucleic acid. Such nucleic acids are used as markers or probes for disease states. For example, a marker is a nucleic acid molecule containing a nucleotide polymorphism, e.g., a point mutation, associated with an iron disorder disease state flanked by wild type HFE sequences. The invention also encompasses nucleic acid molecules that hybridize, preferably under stringent conditions, to a nucleic acid molecule encoding a mutated HFE gene product (or a complementary strand of such a molecule). Preferably the hybridizing nucleic acid molecule is 400 nucleotides, more preferably 200 nucleotides, more

preferably 100, more preferably 50, more preferably 25 nucleotides, more preferably 20 nucleotides, and most preferably 10-15 nucleotides, in length. For example, the nucleotide probe to detect a mutation is 13-15 nucleotides long. The nucleic acids are also used to produce recombinant peptides for generating antibodies specific for mutated HFE gene products. In preferred embodiments, an isolated nucleic acid molecule encodes an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C, as well as nucleic acids the sequence of which are complementary to such nucleic acid which encode a mutant or wild type HFE gene product.

Also within the invention are substantially pure mutant HFE gene products, e.g., an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C. Substantially pure or isolated HFE polypeptides include those that correspond to various functional domains of HFE or fragments thereof, e.g., a fragment of HFE that contains the $\alpha 1$ domain.

Wild type HFE binds to the transferrin receptor and regulates the affinity of transferrin receptor binding to transferrin. For example, a C282Y mutation in the HFE gene product reduces binding to the transferrin receptor, thus allowing the transferrin receptor to bind to transferrin (which leads to increased iron absorption).

The polypeptides of the invention encompass amino acid sequences that are substantially identical to the amino acid sequence shown in Table 2 (SEQ ID NO:2). Polypeptides of the invention are recombinantly produced, chemically synthesized, or purified from tissues in which they are naturally expressed according to standard biochemical methods of purification. Biologically active or functional polypeptides are those which possess one or more of the

biological functions or activities of wild type HFE, e.g., binding to the transferrin receptor or regulation of binding of transferrin to the transferrin receptor. A functional polypeptide is also considered within the scope of the invention if it serves as an antigen for production of antibodies that specifically bind to an HFE epitope. In many cases, functional polypeptides retain one or more domains present in the naturally-occurring form of HFE.

The functional polypeptides may contain a primary amino acid sequence that has been altered from those disclosed herein. Preferably, the cysteine residues in exons 3 and 4 remain unchanged. Preferably the modifications consist of conservative amino acid substitutions. The terms "gene product", "protein", and "polypeptide" are used herein to describe any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation). Thus, the term "HFE polypeptide or gene product" includes full-length, naturally occurring HFE protein, as well a recombinantly or synthetically produced polypeptide that correspond to a full-length naturally occurring HFE or to a particular domain or portion of it.

The term "purified" as used herein refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Polypeptides are said to be "substantially pure" when they are within preparations that are at least 60% by weight (dry weight) the compound of interest. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate

standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

Diagnostic kits for identifying individuals suffering from or at risk of developing an iron disorder are also within the invention. A kit for detecting a nucleotide polymorphism associated with an iron disorder or a genetic susceptibility thereto contains an isolated nucleic acid which encodes at least a portion of the wild type or mutated HFE gene product, e.g., a portion which spans a mutation diagnostic for an iron disorder or hemochromatosis (or a nucleic acid the sequence of which is complementary to such a coding sequence). A kit for the detection of the presence of a mutation in exon 2 of an HFE nucleic acid contains a first oligonucleotide primer which is 5′ to exon 2 and a second oligonucleotide primer is 3′ to exon 2, and a kit for an antibody-based diagnostic assay includes an antibody which preferentially binds to an epitope of a mutant HFE gene product, e.g., an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C, compared to its binding to the wild type HFE polypeptide. An increase in binding of the mutant HFE-specific antibody to a patient-derived sample (compared to the level of binding detected in a wild type sample or sample derived from a known normal control individual) indicates the presence of a mutation which is diagnostic of an iron disorder, i.e., that the patient from which the sample was taken has an iron disorder or is at risk of developing one. The kit may also contain an antibody which binds to an epitope of wild type HFE which contains residue 105, 93, or 65. In the latter case, reduced binding of the antibody to a patient-derived HFE gene product (compared to the binding to a wild type HFE gene product or a gene product derived from a normal control individual) indicates the presence of a mutation which is

diagnostic of an iron disorder, i.e., that the patient from which the sample was taken has an iron disorder or is at risk of developing one.

Individual mutations and combinations of mutations in the HFE gene are associated with varying severity of iron disorders. For example, the C282Y mutation in exon 4 is typically associated with clinical hemochromatosis, whereas other HFE mutations or combinations of mutations in HFE nucleic acids are associated with disorders of varying prognosis. In some cases, hemochromatosis patients have been identified which do not have a C282Y mutation. The I105T and G93R mutations are each alone associated with an increased risk of iron overload (compared to, e.g., the H63D mutation alone), and the presence of both the I105T and H63D mutation is associated with hemochromatosis. Accordingly, the invention includes a method of determining the prognosis for hemochromatosis in a mammal suffering from or at risk of developing said hemochromatosis by (a) detecting the presence or absence of a first mutation in exon 4 in each allele of an HFE nucleic acid, e.g., patient-derived chromosomal DNA, and (b) detecting the presence of a second mutation in exon 2 in each allele of the nucleic acid. The presence of the first mutation in both chromosomes, i.e. an exon 4 homozygote such as a C282Y homozygote, indicates a more negative prognosis compared to the presence of the second mutation in one or both chromosomes, i.e., an exon 2 heterozygote or homozygote. An exon 4 mutation homozygote is also associated with a more negative prognosis compared to the presence of a first mutation (exon 4) in one allele and the presence of the second mutation (exon 2) in one allele, i.e., a compound heterozygote.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is a diagram of the family of proband 1 (HFE genotype H63D/I105T). □ = male, ● = female, ⊘ = deceased, ■ = hemochromatosis phenotype. Proband 1 is indicated by an arrow. Phenotype and genotype data: age in year saturation; % Ftn = serum ferritin concentration. I105 separate chromosomes. The sister of the proband (II, 203) has hyperferritinemia.

Fig. 2 is a diagram of the family of proband 2 (HFE genotype C282Y/G93R). Symbols and abbreviations are the same as those described for Fig. 1. Proband 2 is indicated with an arrow. G93R, C282Y, and wt alleles are known to exist only on separate chromosomes. The father and sister of the proband are being treated for hemochromatosis.

Fig. 3 is a diagram of the family of proband 3 (HFE genotype C282Y/S65C). Symbols and abbreviations are the same as those described for Fig. 1. Proband 3 is indicated with an arrow. S65C, C282Y, and wt alleles are know to exist only on separate chromosomes. Proband 3 also has porphyria cutanea tarda, and her brother (II, 203) has ankylosing spondylitis.

Detailed Description

A proband is the first individual in a family identified to be affected by hemochromatosis. Forward and reverse sequencing of HFE exons 2, 3, 4, and 5, and of portions of HFE introns 2, 4, and 5 was carried out on biological samples taken from twenty hemochromatosis probands who lacked C282Y homozygosity, C282Y/H63D compound heterozygosity, or H63D homozygosity. Four probands had

novel HFE coding region mutations. Probands 1 and 2 were heterozygous for previously undescribed mutations: exon 2, nt 314T→C (314C; I105T), and exon 2, nt 277G→C (277C; G93R), respectively; these probands were also heterozygous for H63D and C282Y, respectively. Probands 3 and 4 were heterozygous for an HFE mutation in exon 2, nt 193A→T (193T; S65C). Twelve other probands did not have an exon 2 HFE exon mutation; four were heterozygous for H63D. In probands 1, 2, 3, and 4, the amino acid substitutions I105T, G93R, and S65C (respectively) occurred on separate chromosomes from those with the C282Y or H63D mutations. In 176 normal control subjects, two were heterozygous for S65C; I105T and G93R were not detected in controls. Nine probands were heterozygous and two probands were homozygous for a base-pair change at intron 2, nt 4919T/C (SEQ ID NO:27). Heterozygosity for a base-pair change in intron 4 (nt 6884T→C) was detected only in probands 3 and 4, both of whom also had S65C and HLA-A32. The intron 2 mutation is not diagnostic of an iron disorder and appears randomly in the population. One proband was heterozygous for a base-pair change at intron 5 (nt 7055A→G).

The data described herein indicate that, in addition to the C282Y and H63D HFE mutations, the HFE exon and intron 5 mutations described herein are diagnostic (and prognostic) of iron disorders.

<u>Pathology of iron overload</u>

Iron plays an essential role in normal growth and development, but in elevated concentrations, iron is a toxic inorganic molecule and is the leading cause of death in children by poisoning. It has been implicated in the pathophysiology of a number of common diseases, e.g., hepatitis, cancer, heart disease, reperfusion injury,

rheumatoid arthritis, diabetes, AIDS, and psychological abnormalities (e.g. depression).

The incidence of cancer (especially liver cancer) rises dramatically in the course of hemochromatosis. Iron, acting alone or in synergy with other environmental agents, catalyzes free radical formation. These free radicals which mediate tissue damage also cause DNA double strand breaks and oncogene activation. Iron may also play a role in the pathogenesis of rheumatic diseases and in predisposition to heart disease. High levels of iron can also cause diabetes with 2% of diabetics being hemochromatosis patients. High levels of iron may also affect the disease progression of many viral diseases. Individuals infected with such viruses as hepatitis (e.g., hepatitis B or C) or HIV should be tested for HFE mutations because of the impact increased iron stores have on the treatment and prognosis of such diseases.

Excessive iron stores and iron deposition is also a major contributing factor in the pathology and treatment of non-valvular heart disease. These conditions include dilated cardiomyopathy cased by deposition of iron in myocardial fibers; myocardial injury the product of anthracycline cardiomyopathy and re-perfusion injury. Increased iron stores may also be a contributing factor in myocardial infarction due to atherosclerosis. Some evidence suggests a significant increase in the incidence of reported heart disease in probands (cardiac symptoms-32%, insulin-dependent diabetes-18%, cardiac arrhythmia-17%, clinically significant coronary artery atherosclerosis-9%, and congestive heart failure-7%. Cardiac complications have been detected in 30% of patients. These include EKG abnormalities, congestive heart failure and cardiac arrhythmias. An increased frequency of HFE mutations in

individuals with porphyria cutanea tarda indicates that HFE mutations may predipose an individual to developing this syndrome.

The effect of iron overload is irreparable damage to vital organs and a multiplicity of associated pathologies described above. The multiplicity of clinical symptoms (and associated pathologies) often causes misdiagnosis of hemochromatosis or failure to diagnose hemochromatosis.

Untreated hemochromatosis is characterized by iron overload of parenchymal cells, which is toxic and the probable cause of various complications including cirrhosis, and liver cancer, arthropathy, hypogonadotropic hypogonadism, marrow aplasia, skin disorders, diabetes mellitus, and cardiomyopathy. There are 1.5 to 2 million active cases in the U.S. of which 40% have progressive liver disease because they have not been properly diagnosed or treated.

In untreated hemochromatosis, iron is universally deposited in the hepatocytes of the liver. The iron is found primarily in the cytoplasm of hepatocytes, and by electron microscopy in lysosomal vacuoles, and in more severe cases iron has also been reported deposited in mitochondria. Other liver toxins such as alcohol, and hepatitis exacerbate the damage caused by the iron deposition. Patients with hemochromatosis are advised not to drink, because of increased liver damage, or to smoke, as iron deposition can also occur in the lungs.

Individuals which are homozygous (and to a lesser extent heterozygous) for an HFE mutation are at risk for developing increased levels of blood lead. Thus, it is important to identify heterozygous as well as homozygous patients.

Identification and detection of mutations in the HFE gene are critical to understanding the general mechanisms of iron disorders and diagnosing iron-related pathologies.

Nucleic acid-based assays for HFE mutations

A biological sample containing RNA or DNA is obtained from an individual and the nucleic acid extracted. Optionally, the nucleic acid is amplified according to standard procedures such as PCR. A nucleic acid polymorphism, e.g, a single base pair polymorphism, is detected using methods well known in the art of molecular biology. For example, a mutation is detected using a standard sequencing assay, nucleic acid hybridization, e.g, using standard Southern, Northern, or dot blot hybridization assay systems and an HFE-specific oligonucleotide probe, restriction enzyme fragment polymorphism analysis, oligonucleotide ligation assay (OLA; Nikerson et al., 1990, Nucl. Acids Res. 87:8923-8927), primer extension analysis (Nikiforov et al., 1994, Nucl. Acids Res. 22:4167-4175), single strand conformation polymorphism (SSCP) analysis, allele-specific PCR (Rust et al., 1993, Nucl. Acids Res. 6:3623-3629), denaturing gradient gel electrophoresis (DGGE), fluorescent probe melting curve analysis (Bernard et al., 1998, Am. J. Pathol. 153:1055-61), RNA mismatch cleavage assay, capillary hybridization, or TaqMan™ assay (PE Applied Biosystems, Foster City, CA). Nucleic acid hybridization assays are also carried out using a bioelectronic microchip technology known in the art, e.g., that described in Sosnowski et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:1119-1123; Cheng et al. 1998, Nature Biotechnology 16:541-546; or Edman et al., 1997, Nucl. Acids Res. 25:4907-4914.

Detection of mutations using antibodies and other HFE ligands

Anti-HFE antibodies are know in the art, e.g., those described by Feder et al., 1997, J. Biol. Chem. 272:14025-14028, or are obtained using standard techniques. Such antibodies can be polyclonal or monoclonal. Polyclonal antibodies can be obtained, for example, by the methods described in Ghose et al., Methods in Enzymology, Vol. 93, 326-327, 1983. An HFE polypeptide, or an antigenic fragment thereof, is used as an immunogen to stimulate the production of HFE-reactive polyclonal antibodies in the antisera of animals such as rabbits, goats, sheep, rodents and the like. HFE antibodies specific for mutated HFE gene products are raised by immunizing animals with a polypeptide spanning the mutation, e.g, a polypeptide which contains the mutations described herein. For example, the entire α1 domain of a mutant HFE gene product is used as an immunogen. Monoclonal antibodies are obtained by the process described by Milstein and Kohler in Nature, 256:495-97, 1975, or as modified by Gerhard, Monoclonal Antibodies, Plenum Press, 1980, pages 370-371. Hybridomas are screened to identify those producing antibodies that are highly specific for an HFE polypeptide containing a mutation characteristic of an iron metabolism abnormality or clinical hemochromatosis. Preferably, the antibody has an affinity of at least about 10^5 liters/mole, preferably at least 10^6 liters/mole, more preferably at least 10^8 liters/mole, and most preferably, an affinity of at least about 10^9 liters/mole.

Antibodies specific for the wild type HFE can also be used to diagnose hemochromatosis or iron metabolism abnormalities. Such antibodies are also useful research tools to identify novel mutations indicative of iron disorders or hemochromatosis. A reduction in binding to a

wild type HFE-specific antibody indicates the presence of a mutation. Antibody binding is detected using known methods. For example, an ELISA assay involves coating a substrate, e.g., a plastic dish, with an antigen, e.g., a patient-derived biological sample containing an HFE gene product. An antibody preparation is then added to the well. Antibodies specific for a mutant HFE gene product bind or fail to bind to a patient-derived sample in the well. Non-binding material is washed away and a marker enzyme e.g., horse radish peroxidase or alkaline phosphatase, coupled to a second antibody directed against the antigen-specific primary antibody is added in excess and the nonadherent material is washed away. An enzyme substrate is added to the well and the enzyme catalyzed conversion is monitored as indicative of presence of the mutation. Antibodies are also labelled with various sizes of colloidal gold particles or latex particles for detection of binding.

The invention employs not only intact monoclonal or polyclonal antibodies, but also an immunologically-active antibody fragment, for example, a Fab or $(Fab)_2$ fragment; an antibody heavy chain, an antibody light chain; a genetically engineered single-chain Fv molecule (Ladner et al., U.S. Patent No. 4,946,778).

Example 1: Selection and Characterization of Subjects

All individuals studied were Caucasians, 18 years of age or older, and from central Alabama. Twenty probands were identified that were either heterozygous for C282Y or H63D, or lacked these mutations. Hemochromatosis is typically diagnosed by detecting elevated saturation of transferrin, with elevated serum ferritin levels, combined with liver biopsy. Each proband patient described below was previously diagnosed to have hemochromatosis by the working diagnostic criterion for hemochromatosis of the American

College of Pathologists (elevated fasting transferrin saturation of greater than 60% saturation for males and greater than 50% saturation for females) on at least two occasions in the absence of other known causes. Probands were interviewed regarding their general medical history, diet (including estimated iron content and ethanol consumption), medicinal iron use, receipt of blood transfusion, prior significant hemorrhage, blood donation for transfusion and/or therapeutic phlebotomy, and pregnancy and lactation. Each proband was also evaluated for viral hepatitis B and C and other hepatic disorders, excess ethanol intake, and hereditary, and acquired anemia. Iron overload was defined as evidence of systemic iron overload demonstrated by otherwise unexplained elevated serum ferritin concentration (≥300 ng/mL in men, ≥200 ng/mL in women), increased hepatic iron content determined using hepatic biopsy specimens, or iron >4 g mobilized by phlebotomy. Complications of iron overload were evaluated and treated, and therapeutic phlebotomy was performed using standard methods. HFE mutation analysis for C282Y and H63D and human leukocyte antigen (HLA) immunophenotyping or molecular typing were performed using known methods. In some family members, HLA haplotyping had been performed previously for other disease associations, or their HLA type could be deduced from analysis of their kinship and HFE genotyping results. Measurement of serum iron and other clinical laboratory parameters and analysis of hepatic biopsy specimens were performed using routine methods. Control subjects (n=176) who were in apparently good health and were unrelated to the hemochromatosis probands were recruited from the general population. Iron parameters were measured and HLA typing was performed in two control

subjects after HFE genotyping revealed that they had the S65C mutation.

Example 2: HFE Gene Analysis

PCR amplification was used to detect mutations. Genomic DNA was prepared from peripheral blood buffy coat or saliva using the QIAmpBlood Kit (QIAGEN, Valencia, CA) or FTA Paper and FTA purification reagent (Fitzco Inc., Maple Plain, MN), respectively. Fragments were amplified from genomic DNA using eLONGase (Life Technologies, Gaithersburg, MD) or HotStarTaq DNA polymerase (QIAGEN, Valencia, CA). Primers used to amplify each exon are shown in Table 3.

Table 4: Human HFE genomic DNA

1 ggatccttta accgaggaga ttattatagc cggagctctg aagcagcaat ctcagttctt
61 gtgatagtga gcaaagaact acaaactaac accaaaatgc aagcttaaag caaagtttat
121 tgaagcacaa taatacactc tgagggacag cgggcttatt tctgcgaagt gaactcagca
181 cttctttaca gagctcaagg tgcttttatg ggtttgtgg ggaggagttg aggtttgggc
241 tgtatctgag tgacaggatg atgttatttg attgaagttt atagctatac aatctaaaat
301 taaactgtgc atggtcttac ctataatttg ttaagaaaag cctcccaggg atgggggggc
361 aaaactgtat gtaaattcta ttataatgat ggcatgatga acttggggtg aacttgaaga
421 caggcttttg tgttgttggg catgtgccac cttagggaat ttccacctgt accctccttt
481 ctctttctcc aggatatttt ggccacagac tttatcataa actccatccc ttagggtggc
541 attagggtag tcttgggcct gaatttaggt gggccagtgg ctgtcttagt gacagccttt
601 ccgctctctt ctgtcatccc ctcccaactg ctaatgtcta actacctaac aattacccat
661 taaatcagtg tgtctggggt taggagcagg cctcaatatg tttaatcatt ctccagataa
721 tcccaatact gtaaagtttg tgaaacactt gtcagataat tcaattatga aggctgtgga
781 acgtgtttca gtaggatcta attggttaat gttatgactt aattaatttg aatcaaaaaa
841 caaaatgaaa aagctttata tttctaagtc aaataagaca taagttggtc taaggttgag
901 ataaaatttt taaatgtatg attgaatttt gaaaatcata aatatttaaa tatctaaagt
961 tcagatcaga acattgcgaa gctactttcc ccaatcaaca acaccccttc aggatttaaa
1021 aaccaagggg gacactggat cacctagtgt ttcacaagca ggtaccttct

gctgtaggag
1081 agagagaact aaagttctga aagacctgtt gcttttcacc aggaagtttt
actgggcatc
1141 tcctgagcct aggcaatagc tgtagggtga cttctggagc catccccgtt
tccccgcccc
1201 ccaaaagaag cggagattta acggggacgt gcggccagag ctggggaaat
gggcccgcga
1261 gccaggccgg cgcttctcct cctgatgctt ttgcagaccg cggtcctgca
ggggcgcttg
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1381 ctagcttttt ctttgcgctt gggagtttgc taactttgga ggacctgctc
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accactgaac
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cggctctgcg
1561 gagtgacttt tggaaccgcc cactcccttc cccaactag aatgctttta
aataaatctc
1621 gtagttcctc acttgagctg agctaagcct ggggctcctt gaacctggaa
ctcgggttta
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ctggaaaatt
1981 aagtatatgt tagttttgaa cgtttgaact gaacaattct cttttcggct
aggctttatt
2041 gatttgcaat gtgctgtgta attaagaggc ctctctacaa agtactgata
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aattcagatt
2221 atataactct tttcaggtta caaagaacat aaataatctg gttttctgat
gttatttcaa
2281 gtactacagc tgcttctaat cttagttgac agtgattttg ccctgtagtg
tagcacagtg
2341 ttctgtgggt cacacgccgg cctcagcaca gcactttgag ttttggtact
acgtgtatcc
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2761 tcagggcaag tcactctggg gctgacactt gagcagagac atgaaggaaa
taagaatgat
2821 attgactggg agcagtattt cccaggcaaa ctgagtgggc ctggcaagtt
ggattaaaaa

2881 gcgggttttc tcagcactac tcatgtgtgt gtgtgtgggg ggggggcgg cgtgggggtg
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10501 gaaaagctat aaaatgaata caattaaagc tgttatttaa ttagccagtg aaaaactatt
10561 aacaacttgt ctattacctg ttagtattat tgttgcatta aaaatgcata tactttaata
10621 aatgtacatt gtattgtata ctgcatgatt ttattgaagt tcttgttcat cttgtgtata
10681 tacttaatcg ctttgtcatt ttggagacat ttattttgct tctaatttct ttacattttg
10741 tcttacggaa tattttcatt caactgtggt agccgaatta atcgtgtttc ttcactctag
10801 ggacattgtc gtctaagttg taagacattg gttattttac cagcaaacca ttctgaaagc
10861 atatgacaaa ttatttctct cttaatatct tactatactg aaagcagact gctataaggc
10921 ttcacttact cttctacctc ataaggaata tgttacaatt aatttattag gtaagcattt
10981 gttttatatt ggttttattt cacctgggct gagatttcaa gaaacacccc agtcttcaca
11041 gtaacacatt tcactaacac atttactaaa catcagcaac tgtggcctgt taattttttt
11101 aatagaaatt ttaagtcctc attttctttc ggtgtttttt aagcttaatt tttctggctt
11161 tattcataaa ttcttaaggt caactacatt tgaaaaatca aagacctgca ttttaaattc
11221 ttattcacct ctggcaaaac cattcacaaa ccatggtagt aaagagaagg gtgacacctg
11281 gtggccatag gtaaatgtac cacggtggtc cggtgaccag agatgcagcg ctgagggttt
11341 tcctgaaggt aaaggaataa agaatgggtg gaggggcgtg cactggaaat cacttgtaga
11401 gaaaagcccc tgaaaatttg agaaaacaaa caagaaacta cttaccagct atttgaattg
11461 ctggaatcac aggccattgc tgagctgcct gaactgggaa cacaacagaa ggaaaacaaa
11521 ccactctgat aatcattgag tcaagtacag caggtgattg aggactgctg agaggtacag
11581 gccaaaattc ttatgttgta ttataataat gtcatcttat aatactgtca gtattttata
11641 aaacattctt cacaaactca cacacattta aaaacaaaac actgtctcta aaatccccaa
11701 atttttcata aactcagttt taaactaact ttttttcaaa ccacaatctg atttaacaat
11761 gactatcatt taaatatttc tgactttcaa attaaagatt ttcacatgca ggctgatatt
11821 tgtaattgtg attctctctg taggctttgg gtataatgtg ttcttttcct tttttgcatc
11881 agcgattaac ttctacactc taacatgtag aatgttacta caatattaaa gtattttgta
11941 tgacaatttt atttgaaagc ctaggatgcg ttgacatcct gcatgcattt attacttgat
12001 atgcatgcat tctggtatct caagcattct atttctgagt aattgtttaa

ggtgtagaag
12061 agatagatat ggtggatttg gagttgatac ttatatattt tctatttctt
ggatggatga
12121 atttgtacat taaaagtttt ccatgg

(SEQ ID NO:27; GENBANK® Accession No. Z92910)

Exon 1 spans nt 1028-1324, inclusive; exon 2 spans nt 4652-4915, inclusive; exon 3 spans nt 5125-5400, inclusive; exon 4 spans nt 6494-6769, inclusive; exon 5 spans nt 6928-7041, inclusive; exon 6 spans nt 7995-9050, inclusive, and exon 7 spans nt 10206-10637, inclusive. Intron 4 spans nt 6770-6927, inclusive, and intron 5 spans nt 7042-7994, inclusive.

Total RNA for the RT-PCR was prepared from 1.5 mL of whole blood using the RNeasy Blood Kit (QIAGEN, Valencia, CA). Total messenger RNA encoding the HFE gene was transcribed and amplified with the primers shown above using standard methods, e.g., the Superscript ONE-STEP RT- PCR System (Life Technologies, Gaithersburg, MD). The amplified product was directly subcloned into the pCR2.1-TOPO vector and transfected into TOP 10 bacteria (Invitrogen, Carlsbad, CA). Plasmid DNAs isolated from the subcloning were prepared with the UltraClean Mini Prep Kit (Mo Bio, Solana Beach, CA) and sequenced.

DNA sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) and analyzed on an ABI Prism 377.

To detect mutations in exon 2 of the HFE gene, the genomic DNA of probands and normal control subjects were amplified and subjected to a dot blot hybridization assay. 1.0 μl of each resulting PCR product was then applied to a Magna Graph nylon membrane (MSI, Westboro, MA). The membranes were treated with 0.5 N NaOH/1.5 M NaCl to denature the DNA, neutralized with 0.5 M Tris-HCl

(pH 8.0)/1.5 M NaCl, and rinsed with 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The DNAs were fixed on the membrane by UV irradiation using a Stratalinker 1800 (Stratagene, Inc., La Jolla, CA). The ECL 3'-oligolabelling and detection system (Amersham, Arlington Heights, IL) was used for synthesis of labeled oligonucleotide probes, hybridization, and signal detection. The oligonucleotide sequences used to detect each point mutation were (substituted bases are shown as upper case letters):

Table 5: Oligonucleotide Probes

Point Mutation	Oligonucleotide
G93R mutation	gtctgaaaCggtgggat (SEQ ID NO:28)
I105T mutation	acttctggactaCtatgg (SEQ ID NO:29)
S65C mutation	atcatgagTgtcgccgt (SEQ ID NO:30)

For signal detection, each oligonucleotide was labeled with fluorescein-11-dUTP using terminal deoxynucleotidyl transferase according to the manufacturer's instructions (Amersham Ltd., Arlington Heights, IL). The membranes were prehybridized in 5 × SSC, 0. 1 % Hybridization buffer component, 0.02% SDS, 5% LiquidBlock at 42°C for approximately 2 hours. Labelled oligonucleotide probes were added to individual bags containing the membranes and prehybridization buffer and incubated at 42°C overnight. The blots were washed twice with 5 × SSC, 0.1 % SDS for 5 minutes at room temperature. Stringency washes for hybridization with oligonucleotides having the sequence of SEQ ID NO: 30 or 28 were performed twice in 0. 2 × SSC/0.1%

SDS for 15 minutes at 42°C. Membranes probed with an oligonucleotide having the sequence of SEQ ID NO:29 was washed twice under less stringent conditions (0.5 × SSC/0.1% SDS, 15 minutes at 42°C). Detection of a fluorescent signal was performed according to standard methods.

Example 3: Characterization of Probands

The mean age of the twenty probands was 44 ± 11 years (range 27-62 years); thirteen (65.0%) were men and seven (35.0%) were women. Eleven had iron overload. One had hepatic cirrhosis, two had diabetes mellitus, four had arthropathy, and two had hypogonadotrophic hypogonadism. One proband also had hereditary stomatocytosis, another had beta-thalassemia trait, a third had ethanol intake >60 g daily, and a fourth had porphyria cutanea tarda. No proband had evidence of excess oral or parenteral iron intake, or of viral hepatitis B or C. At diagnosis of hemochromatosis, evaluation for common HFE mutations revealed that eleven probands were C282Y heterozygotes, five were H63D heterozygotes, and four did not inherit C282Y or H63D.

The mean age of the initial 176 control subjects was 52 ± 15 years (range 18-86 years); 79 (44.9%) were men and 97 (55.1%) were women. There was no significant difference in the mean ages of men and women. Frequencies of HFE genotypes among the control subjects are shown in Table 6. These values are similar to those previously reported from normal persons from the same geographic area.

Table 6. Frequencies of *HFE* Genotypes in Alabama Subjects.

HFE Genotype	Hemochromatosis Probands with "Atypical" *HFE* Genotypes, % *(n)*	Normal Control Subjects, % *(n)*
wt/wt	15.00 *(3)*	60.23 *106)*
C282Y/wt	45.00 *(9)*	13.06 *(23)*
H63D/wt	20.00 *(4)*	15.34 *(27)*
S65C/wt	5.00 *(1)*	1.14 *(2)*
C282Y/S65C	5.00 *(1)*	0
C282Y/G93R	5.00 *(1)*	0
H63D/1105T	5.00 *(1)*	0
H63D/C282Y	0	6.82 *(12)*
H63D/H63D	0	3.41 *(6)*

Results are expressed as percentage *(n)*. The wild-type (wt) allele was defined as the *HFE* configuration in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.

Example 4: Identification of novel HFE Mutations in Hemochromatosis Probands

The following novel mutations (missense mutations) were identified in probands 1 and 2: exon 2, nt 314T→C (I105T), and exon 2, nt 277G→C (G93R), respectively (Table 7; Figs. 1 and 2). Probands 3 and 4 had a S65C mutation The S65C mutation has been observed in hemochromatosis patients but has not been deemed to be indicative of a disease state. In contrast, the data presented herein indicate that the S65C mutation is diagnostic of a disease state. This result is surprising in view of earlier observations. Other than C282Y or H63D, no HFE exon mutations were detected in the remaining sixteen of the twenty probands (Table 6). Nine probands were heterozygous for a base-pair change at intron 2, nt 4919T/C (SEQ ID NO:27); two probands were homozygous for this base-pair change. Heterozygosity for a base-pair change in intron 4 (nt 6884T→C) was detected only in probands 3 and 4, both of whom also inherited S65C. One proband was heterozygous for a base-pair change at intron 5, nt 7055A→G.

Using dot blot methodology, heterozygosity for the S65C mutation was detected in two of 176 normal control subjects (Table 6). The G93R or I105T mutations were not detected in normal control subjects (Tables 6 and 8).

Example 5: Association of Novel HFE Coding Region Mutations to C282Y and H63D and HFE Intron Alleles

In proband 1, two mutations of exon 2 (H63D and I105T) were detected. After subcloning the genomic fragment, the subclones revealed that these mutations occurred on separate chromosomes; this observation was confirmed by family studies indicating segregation of I105T

Table 7. Phenotypes and Uncommon *HFE* Genotypes in Alabama Subjects*

Subject†	Age (years), Sex	*HFE* Genotype	HLA Type	Transferrin Saturation, %	Serum Ferritin, ng/mL	Hepatocyte Iron Grade	Phlebotomy, Units
Proband 1	52 M	H63D/I105T	A2, 3; B7,7	62	868	2+	20
Proband 2‡	40 M	C282Y/G93R	A2, 3; B7, 62	78	861	4+	34
Proband 3§	47 F	C282Y/S65C	A2, 32; B8, 44; Bw4, 6; Cw5, 7	90	281	3+	37
Proband 4**	81 F	S65C/wt	A2, 32; B14, 62	100	5,135	N.D.	37
Normal Control 1	28 M	S65C/wt	A2, 31; B35, 60	28	141	N.D.	N.D.
Normal Control 2	69 M	S65C/wt	A24, 26; B8, B37; Bw4, 6; Cw6, 5 (or 7)	42	747	2+	N.D.

*Serum transferrin saturation, serum ferritin concentration, and percutaneous hepatic biopsy were performed before therapeutic phlebotomy was initiated. Reference ranges for these parameters are 15 - 45%; 20 - 300 ng/mL (men) and 20 - 200 ng/mL (women); and 0 -1+, respectively. Iron depletion (serum ferritin ≤ 20 ng/mL) was induced by removing the indicated numbers of units of blood. None of these persons had evidence of hepatic cirrhosis, diabetes mellitus, hemochromatosis-associated arthropathy, hypogonadotrophic hypogonadism, other endocrinopathy, or cardiomopathy. N.D. = not done. The mutations indicated are exon 4, nt 845G→A (C282Y); exon 2, nt 187C→G (H63D); exon 2, nt 314T→C (I105T); exon 2, nt 277G→C (G93R); and exon 2, nt 193A→T (S65C). The wild-type (wt) allele was defined as an *HFE* allele in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.

†Countries of origin: Probands 1 and 2, England; Proband 3, Wales, England, and Americas (Cherokee); Proband 4, England and Ireland; Normal Control 1, England; Normal Control 2, The Netherlands.

‡The father and sister of Proband 2 are presently undergoing therapy for hemochromatosis and iron overload, but their clinical and genetic data were unavailable.

§Proband 3 had porphyria cutanea tarda alleviated with therapeutic phlebotomy.

**Proband 4 had hereditary stomatocytosis unaffected by phlebotomy treatments. 37 units of blood were removed by phlebotomy before treatment was discontinued due to stroke apparently unrelated to anemia or iron overload (post-treatment serum ferritin 1,561 ng/mL). Her 59 year-old daughter (who does not have hereditary stomatocytosis) had transferrin saturation 42%, serum ferritin 62 ng/mL, HLA type A1, 32; B14, 15; Bw4, 6; Cw3, 8, and *HFE* genotype S65C/H63D. These data permitted assignment of the S65C mutation in this family to a haplotype carrying HLA-A32; linkage of S65C and HLA-A32 was also observed in the family of Proband 3.

Table 8. Frequencies of *HFE* Alleles in Alabama Subjects.

	wt*	C282Y	H63D	S65C†	I105T	G93R
Hemochromatosis Probands with "Atypical" *HFE* Genotypes (n = 20)	0.500	0.275	0.125	0.050	0.025	0.025
Normal Control Subjects (n = 176)	0.750	0.099	0.145	0.006	‡	‡

The wild-type (wt) allele was defined as an *HFE* allele in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.

†S65C was detected in 2 of 22 (0.091) proband chromosomes and in 2 of 266 (0.0075) control chromosomes that did not bear the C282Y, H63D, S65C, I105T, or G93R mutation.

‡Based on this data set, the frequency of the I105T and G93R *HFE* alleles is estimated to be < 0.0028, respectively.

and H63D (Fig. 1). In proband 2 (HFE genotype C282Y/G93R), RT-PCR analysis (with subsequent subcloning and sequencing) revealed that these HFE mutations occurred on separate chromosomes. Family studies of proband 3 (HFE genotype C282Y/S65C) indicated that the C282Y and S65C HFE alleles segregated independently, establishing their occurrence on separate chromosomes (Table 7, Fig. 3).

In proband 1 (HFE genotype H63D/I105T), the I105T mutation was co-inherited with HLA-A3, B7. In probands 3 and 4 and their respective families, S65C was inherited on the same chromosome as HLA-A32, indicating that HLA-A32 is a marker for chromosomes bearing the S65C mutation, and individuals with HLA-A32 have an increased risk for developing hemochromatosis. The G93R mutation is associated with HLA-A2, and individuals with that haplotype have an increased risk for developing hemochromatosis. The I105T mutation is associated with HLA-A3, e.g., HLA-A3, B7, and individuals with that haplotype have an increased risk for developing hemochromatosis. Among twenty probands tested, the nucleotide polymorphism in intron 4 (nt 6884T→C) was detected in probands 3 and 4, both of whom also had S65C. Subjects that tested positive for the S65C mutation all were found to have the intron 4 (6884T→C) mutation, including two probands (3 and 4), their families, and two normal controls.

Example 6: HFE Coding Region Mutations and Clinical Phenotype

The I105T and G93R mutations were associated with a hemochromatosis clinical phenotype in probands 1 and 2 who also inherited H63D and C282Y, respectively. Proband 3 had clinical evidence of hemochromatosis, iron overload, and porphyria cutanea tarda associated with compound heterozygosity for C282Y and S65C. Proband 4 had severe iron overload associated with heterozygosity for S65C and

co-inheritance of hereditary stomatocytosis (Table 7). The sister of proband 1 (HFE genotype I105T/wt) was not completely evaluated for hyperferritinemia (Fig. 1). Otherwise, family members of probands who were heterozygous for novel HFE mutations described herein had little or no evidence of abnormal iron parameters, a hemochromatosis phenotype, or of iron overload (Table 7 and 9; Figs. 1 and 3). Normal Control 1 who had HFE genotype S65C/wt had a

Table 9. Hemochromatiosis (HC) Family study/patent

Subject/Age/Sex	HLA Type	exon 2	exon 4	intron 4	Tf sat**	Ftn**	Diagnosis/Hepatocyte
				5636bp	%	ng/ml	Iron grade
Proband 1/57M (201)	A2,3;B7,7	H63D/H,1105T/1	Wt	T	62	868	HC/2+
brother/45M(204)		H63D/H	Wt	T*	31	186	
sister/50F(203)	A3,3:B7,7	1105T	Wt*	T*	37	576	
daughter/31F(301)	A32,68;B7,44	1105T/1	Wt*	T*	31	56	
son/27M(302)	A2,68;B7,44	H63D/H	Wt*	T*	33	44	
Proband 2/40M	A2,3;B7,62	G93R/G	C282Y/C	T	78	861	HC/4+
Father		Wt	C282Y/Y*	T*			HC
Sister		G93R/G	C282Y/C*	T*			HC
Proband 3/47(201)	A2,32;B8,44	S65C/S	C282Y/C	T/C	90	281	HC/3+
brother/45M(202)	A2,32;B44,51	S65C/S	Wt	T/C	33	42	
mother/81F(102)	A2,2;B8,51	Wt	C282Y/C	T*	NT	NT	
sister/33F(204)	A2,7;B27,51	Wt	Wt	T*	NT	NT	
brother/35M(203)	A2,7;B27,51	Wt	Wt*	T*	NT	NT	
sister		Wt	C282Y/C*	T*			
sister		S65C/S	Wt*	T/C*			
Proband 4/81F	A2,32;B14,62	S65C/S	Wt	T/C	100	S135	HC+stomatocytosis
daughter/59"	A1,32;B14,15	H63D/H,S65C/S	Wt*	T/C	42	62	
Control 1/28M	A2,31;B35,60	S65C/S	Wt	T/C	28	141	
Control 2/69M	A24,26;B8,37	S65C/S	Wt	T/C	42	747	2+

*RE cut
**normal (15-45%)
***20-300ng/ml(men)
2C-200ng/ml(women)

normal iron phenotype (Table 7). Normal Control 2, who also had the HFE genotype S65C/wt, had hyperferritinemia and mildly increased stainable hepatocellular iron deposition, but had no symptoms or other objective findings attributable to iron overload (Table 7). These data indicate that S65C heterozygosity is associated with abnormal iron parameters.

<u>Example 7: HLA gene linkage</u>

In the family of proband 1, the I105T mutation was linked to HLA-A3, B7, markers which are often linked to the C282Y mutation and its ancestral haplotype. HLA-A3, B7 is also significantly more common among C282Y-negative hemochromatosis probands than in normal control subjects tested. S65C was linked to HLA-A32 in probands 3 and 4 (and their respective families). The base-pair change in intron 4 (nt 6884T→C) was detected only in probands who inherited the S65C mutation. These data indicate that an intron 4 mutation (nt 6884→C) is a marker for chromosomes bearing the S65C HFE allele. Three of four probands who inherited mutated HFE exon 2 mutations described herein also inherited the C282Y or H63D mutations on separate chromosomes. In a fourth proband, the co-inheritance of S65C heterozygosity and hereditary stomatocytosis was associated with severe iron overload.

Altered interactions of transferrin receptor, transferrin, and C282Y and H63D mutant HFE protein contribute to the pathology of hemochromatosis. The S65C, G93R, and I105T mutations are located within the α1 domain: in the α1 helix of the HFE class I-like heavy chain (I105T and G93R), and at the tip of the A chain loop of the β-pleated sheet (S65C). These mutations affect the overall structure of the HFE gene product, and specifically affect the salt bridge between residues H63 and D95. The I105T substitution also inhibits proper folding of the α1 domain

of the HFE gene product, and specifically affects the hydrophobicity of the hydrophobic F pocket.

Other embodiments are within the following claims.